



Antiparasitic Effect of Copper Alloy Surface on *Cryptocaryon irritans* in Aquaculture of *Larimichthys crocea*

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ABSTRACT Copper and alloys containing >60% copper by weight are antimicrobial. In aquaculture, copper alloys are used as part of corrosion-resistant cages or as part of copper coating. To test whether a copper alloy surface prevents the outbreak of parasitosis in the aquaculture of *Larimichthys crocea*, we covered the bottom of the aquaculture tank with sheets of copper alloy containing 74% to 78% copper, and we cultured *L. crocea* juveniles that had been artificially infected with the protozoan parasite *Cryptocaryon irritans*. Our results showed that these copper alloy sheets effectively blocked the infectious cycle of *C. irritans* within a 1-week period and significantly reduced the number of *C. irritans* trophonts and tomites, thereby decreasing the mortality rate of *L. crocea*. In *in vitro* assays, the cytoplasmic membranes of protomonts disintegrated and the cytoplasm overflowed after just 5 minutes of contact with copper alloy surfaces. Although the same cytoplasmic membrane disintegration was not observed in tomites, the tomites completely lost their capacity for proliferation and eventually died following direct contact with copper alloy sheets for 1 h; this is likely because *C. irritans* tomites took in >100 times more copper ions following contact with the copper alloy sheets than within the control aquaculture environment. Exposure to copper alloy sheets did not lead to excessive heavy metal levels in the aquacultured fish or in the culture seawater.

IMPORTANCE *Cryptocaryon irritans*, a parasitic ciliate that penetrates the epithelium of the gills, skin, and fins of marine fish, causes acute suffocation and death in cultured fish within days of infection. Much of the existing research centers around the prevention of *C. irritans* infection, but no cure has been found. Studies demonstrate that copper has strong antimicrobial properties, and fish grown in copper-containing cages have lower rates of *C. irritans* infection, compared to those grown in other currently used aquaculture cages. In this study, we found that an alloy containing 74% to 78% copper by weight effectively killed *C. irritans* cells and prevented cryptocaryoniasis outbreaks within a 1-week period. These findings offer a new perspective on the prevention and control of cryptocaryoniasis.

KEYWORDS antiparasitic effect, copper alloy surface, *Cryptocaryon irritans*, *Larimichthys crocea*, protomont, tomit

Cryptocaryon irritans is a eukaryotic ciliate that can parasitize the skin, gills, and fin epithelium of marine teleosts, and it is the cause of "marine white spot disease" (1). This disease occurs annually in China's eastern and southern coastal areas, where high-density aquaculture is located, causing acute suffocation and fish death within days. This parasite has become the principal parasitic killer of aquacultured fishes in

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these areas and leads to huge economic losses. Therefore, research on methods to prevent and/or to cure cryptocaryoniasis is currently an area of great interest (2).

Much of the existing research is aimed at finding an efficient and environmentally friendly way to cure or to prevent cryptocaryoniasis. However, some of the challenges include the difficulty of vaccine development (3, 4), the toxicity of chemical agents to fish, environmental pollution by residual chemical agents (5), and asynchronous development of *C. irritans* (1). The life cycle of *C. irritans* includes the trophont, protomont, tomont, and theront stages. The trophonts parasitize the surface of the host and naturally shed from the body of parasitized fish, 2 to 4 days postinfection, in the form of protomonts, which slowly crawl along the bottom of the culture tank for 4 to 6 hours and secrete cyst wall materials to form the reproductive tomonts. Protoplasts in the tomonts can form up to 300 tomites/theronts after several consecutive unequal divisions, and the infective theronts break out of the cyst and swim quickly in the seawater to find fish to reinfect within a few hours (1). The life cycle of *C. irritans* is only 1 week at 28°C (6), and the main reason for *C. irritans*-induced death is large-scale reinfection (7). Since the protomonts and tomonts attach to the bottom of the aquaculture tank, the current view is that, by increasing the isolation of protomonts and tomonts or by preventing the protomonts and tomonts from developing, the life cycle of *C. irritans* could be blocked effectively and disease outbreaks could be controlled. Regularly changing aquaculture tanks would isolate the fish from the pathogens (8), but this practice is stressful for the fish. Regularly replacing the bottom place mat would be somewhat effective (9), but the associated labor costs could be exceedingly high. For these reasons, it would be a significant step forward in aquaculture management if the protomonts and tomonts could be killed using a nonpolluting and cost-effective method.

Copper has strong antimicrobial properties and is commonly used in the manufacture of hospital door handles, touch pads, telephone buttons, toilets, and other surfaces where microbial growth must be controlled (10, 11). In 2008, more than 300 copper surfaces were registered by the Environmental Protection Agency as antimicrobial materials (12). Studies have confirmed that contact with copper surfaces kills *Staphylococcus aureus*, *Escherichia coli*, and most other bacteria within hours or even seconds, due to the combinatory effects of copper-ion-induced reactive oxygen species (ROS) generation (13), lipid peroxidation (14), damage to cell membrane integrity (15), DNA degradation (16), aberrant protein expression (17), inactivation of metalloproteins (18), and other factors. In marine fish farming, copper is used for corrosion-resistant cages, and copper coating is also used for its anticorrosive, antibacterial, and antiadhesive properties (19). It has been shown that copper ion concentrations in farmed fish and mussels do not exceed the established limits for food products set forth by the Food and Agriculture Organization (FAO) of the United Nations (20). Anecdotally, some aquaculturists also find that fish grown in copper-containing cages have a lower risk of *C. irritans* infection, compared to those grown in other current aquaculture cages. Whether copper-containing materials show similar effects in land-based aquaculture (more specifically, whether copper-containing surfaces can directly kill *C. irritans* and other eukaryotic parasites) has not been reported.

Larimichthys crocea is a major fish species in marine aquaculture and is greatly affected every year by outbreaks of cryptocaryoniasis. In this study, we covered the bottom of *L. crocea* aquaculture tanks with sheets of copper alloy containing 74% to 78% copper and cultured *L. crocea* juveniles that had been artificially infected with *C. irritans*. Our results show, for the first time, that a copper-containing surface effectively prevents *C. irritans* reinfection and cryptocaryoniasis outbreak within a 1-week period.

RESULTS

Copper surfaces prevent mass deaths of *L. crocea* from cryptocaryoniasis. Two groups of fish (groups B and C) were infected with *C. irritans* at a dose of 20 theronts/g of fish. The tank bottoms for group C, but not those for group B, were covered with copper alloy sheets. Uninfected fish were used as the control group (group A), and their

TABLE 1 Surviving numbers of *Cryptocaryon irritans*-infected *Larimichthys crocea* fish following copper alloy surface exposure

Group ^a	No. of surviving fish after ^b :									
	1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d	9 d	15 d
A	30	30	30	29.00 ± 1.00	28.00 ± 1.73	27.67 ± 2.31	27.33 ± 2.89	27.00 ± 3.46	26.67 ± 3.21	26.67 ± 3.21
B	30	30	30	29.00 ± 1.73	28.67 ± 2.31	28.67 ± 2.31	1.33 ± 2.31	0.00	0.00	0.00
C	30	30	30	27.00 ± 1.00	26.00 ± 1.73	25.67 ± 2.31	25.67 ± 2.31	25.67 ± 2.31	25.67 ± 2.31	25.67 ± 2.31
P				0.171	0.289	0.337	<0.0001			-

^aGroup A, uninfected fish and their tank bottoms with no copper alloy covering were used as the control group; group B, infected fish and their tank bottoms were not covered with copper alloy sheets; group C, infected fish and their tank bottoms were covered with copper alloy sheets. Values are mean ± SD (*n* = 3).

^bd, day(s).

tank bottoms were not covered with copper alloy sheets. There were no mass deaths of fish in any of the three groups from day 1 to day 6, and no significant difference in survival rates between the three groups was detected (*P* > 0.05) (Table 1). The survival rate of non-copper-exposed infected fish (group B) decreased by 91.14% on day 7 and was significantly lower than those of the other two groups (*P* < 0.05). On day 8, all of the infected fish in group B had died, while the survival rate for the infected fish in the copper-exposed group (group C) was essentially the same as that for the uninfected control group, remaining above 85% (*P* > 0.05) until the end of the experiment on day 15.

Copper surfaces decrease the number of *C. irritans* cells and prevent the outbreak of cryptocaryoniasis. Relative infection intensity (RII) reflects the number of trophonts on the left fin per gram of fish, which represents the intensity of infection by *C. irritans*. The numbers of trophonts (RII) for both the non-copper-exposed infected fish (group B) and the copper-exposed infected fish (group C) showed two peaks over time (Table 2). The first peak appeared on day 2 after infection, and no significant difference in RII between group B and group C was detected (*P* > 0.05). On days 3 and 4, with the maturation of *C. irritans* cells, the trophonts fell off the fish naturally and the numbers of trophonts in both groups decreased significantly. On days 5 through 7, however, with *C. irritans* multiplication and reinfection, the RII observed in the non-copper-exposed infected fish (group B) spiked again to a level that was approximately 100 times higher than the first peak. Meanwhile, only small numbers of trophonts were observed on infected fish in the tanks containing copper alloy surfaces (group C). The concentration of trophonts observed was far lower than that at the beginning of the experiment, with all of the trophonts being eliminated by day 7.

After maturation, trophonts leave the host to form tomonts. In both non-copper-exposed infected fish (group B) and copper-exposed infected fish (group C), the numbers of tomonts (relative tomont number [RTN]) and those of trophonts (RII) changed in the same direction (Table 2). Far fewer tomonts were collected in both infected groups on day 1. The second peak in group B emerged on day 6 and was far higher than the first peak. In contrast, hardly any protomonts or tomonts were found

TABLE 2 Decreased numbers of *Cryptococcus irritans* cells associated with fish in the presence of copper sheets

Measurement and group ^a	No. of <i>C. irritans</i> cells after ^b :									
	1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d	9 d	15 d
RII (trophonts/g fish)										
A	—	—	—	—	—	—	—	—	—	—
B	0.89 ± 1.13	1.28 ± 0.54	0.06 ± 0.10	0.22 ± 0.38	21.72 ± 11.80	149.61 ± 29.59	23.17 ± 40.13	—	—	—
C	0.78 ± 0.10	1.78 ± 0.19	—	—	0.28 ± 0.48	0.11 ± 0.19	—	—	—	—
RTN (tomonts/g fish)										
A	—	—	—	—	—	—	—	—	—	—
B	0.37 ± 0.35	6.18 ± 2.07	0.42 ± 0.24	0.19 ± 0.08	0.05 ± 0.08	28.74 ± 24.45	17.26 ± 29.90	—	—	—
C	0.09 ± 0.16	4.68 ± 2.61	—	—	—	0.18 ± 0.30	—	—	—	—

^aGroup A, uninfected fish and their tank bottoms with no copper alloy covering were used as the control group; group B, infected fish and their tank bottoms were not covered with copper alloy sheets; group C, infected fish and their tank bottoms were covered with copper alloy sheets. —, no trophonts, protomonts, or tomonts were detected. Values are mean ± SD (*n* = 3).

^bd, day(s).

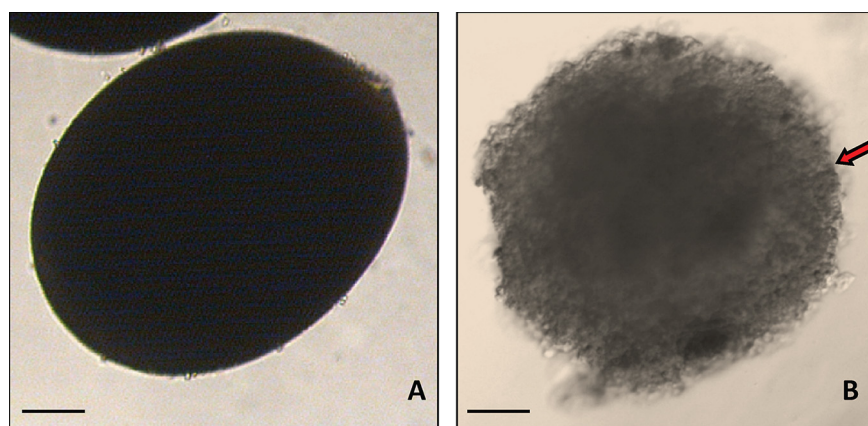


FIG 1 Microstructural images of *C. irritans* protomonts. (A) Protomonts were collected from polystyrene surfaces and used as the control. (B) Protomonts collected from copper alloy surfaces (group C, infected fish exposed to copper sheets) lost their adhesion and showed disintegration and loss of the cytoplasmic membrane (red arrow). Scale bars, 50 μ m.

in group C after day 3; some reemerged on day 6 but rapidly disappeared. Most of the cells collected from copper-exposed infected fish (group C) lost their adhesion and showed disintegration of their cytoplasmic membranes and leakage of their cytoplasm (Fig. 1). No trophonts or tomonts were observed throughout the duration of the experiment in the uninfected control group (Table 2).

In vitro contact killing assay on copper surfaces. *C. irritans* protomonts and tomonts always attach themselves to the substrate of the aquaculture tank. *In vitro* studies showed that the copper surfaces killed both the protomonts and the tomonts through direct contact (Table 3 and Fig. 2 and 3). The mortality rates of both protomonts and tomonts increased significantly with increased contact time. Nine percent of protomonts died after only 5 min of direct contact with the copper surfaces, and leaky plasma membranes were observed (Fig. 2). Similarly, 10% of tomont deaths occurred after 20 min of direct contact with copper surfaces. One hundred percent of both protomonts and tomonts were killed after 60 min of direct contact with the copper surfaces (Table 3). After 2 more weeks of incubation in a beaker with no copper surfaces, the protoplasm of the dead tomonts began to shrink, and the gap between the cyst wall and the plasma membrane increased (Fig. 3). Meanwhile, the polystyrene surface in the control group showed no antiparasitic effects (Fig. 2 and 3). The tomonts were also treated for 0.5 to 12 h using different concentrations of copper sulfate, and we found that treating the tomonts with 1 mmol/liter Cu^{2+} for 0.5 or 1 h or with 0.25 mmol/liter Cu^{2+} for 6 h could effectively kill them. On an agarose gel, DNA purified from *C. irritans* migrated with an apparent molecular size of 10 kDa. None of the tested DNA samples exhibited pronounced smearing effects, suggesting that no DNA degradation took place (Fig. 4).

Copper and zinc concentrations in tomonts, culture seawater, and the fish body. After an *in vitro* assay that placed tomonts in direct contact with copper surfaces for 0.5 h or 1 h, both copper and zinc contents in the tomonts increased significantly ($P < 0.05$) (Table 4). The copper content increased to 124 times that of the control group at 0.5 h and 136 times that of the control group at 1 h, while the zinc content

TABLE 3 Mortality rates of *Cryptocaryon irritans* cells following copper alloy surface treatment, with time after direct contact

Form	Mortality rate (%) ^a							
	3 min	5 min	10 min	15 min	20 min	30 min	45 min	60 min
Protomonts	0.00 \pm 0.00 E	9.00 \pm 10.82 E	31.67 \pm 16.50 DE	64.67 \pm 16.86 CD	81.00 \pm 16.46 BC	94.33 \pm 5.13 AB	95.67 \pm 4.04 AB	100.00 \pm 0.00 A
Tomonts	0.00 \pm 0.00 D	0.00 \pm 0.00 D	0.00 \pm 0.00 D	0.00 \pm 0.00 D	10.00 \pm 5.00 D	65.00 \pm 5.00 C	91.7 \pm 2.89 B	100.00 \pm 0.00 A

^aThe capital letters identify the pairs of values that are statistically significantly different at different time points ($P < 0.05$, Tukey's multiple comparisons). Values are mean \pm SD ($n = 3$).

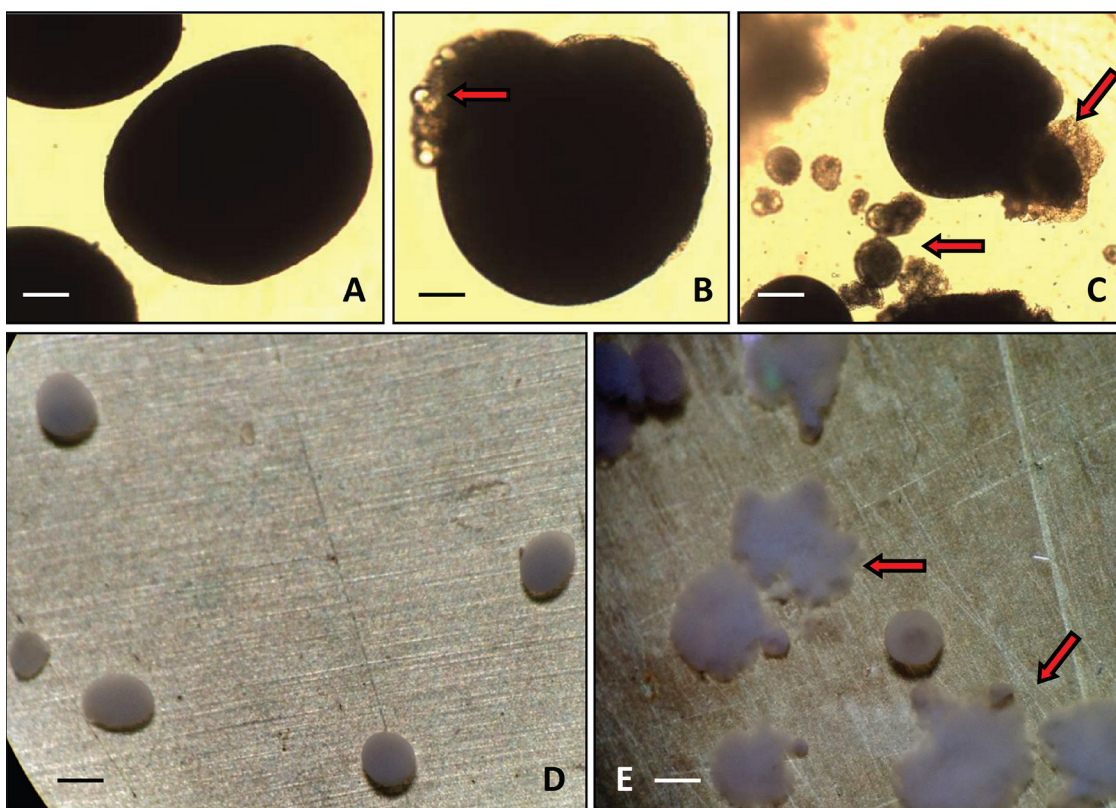


FIG 2 *In vitro* microstructural images of *C. irritans* protomonts. (A) Protomonts were spread on a polystyrene surface and used as the control. (B, C, and E) The cytoplasmic membranes of protomonts disintegrated and the cytoplasm overflowed (arrows) after just 5 min (B), 15 min (C), and 20 min (E) of contact with copper alloy surfaces. (D) Protomonts in contact with copper alloy sheets for 0 min are also shown. Scale bars, 50 μm (A, B, and C) or 200 μm (D and E).

increased to 2.43 times that of control group at 0.5 h and 3.69 times that of the control group at 1 h.

Copper concentrations in aquaculture seawater and the entire *L. crocea* body were measured and compared between the infected group in the presence of copper surfaces (group C) and the control group with no exposure to copper surfaces (group A). In group C, the copper concentration of the seawater was 3.57 $\mu\text{g/liter}$, significantly higher than that of the control group (0.02 $\mu\text{g/liter}$) ($P < 0.05$) but still lower than that specified in seawater quality standard GB 3097-1997, grade I (5 $\mu\text{g/liter}$) (21). The copper concentrations found in whole fish in the copper-exposed group and the control group were 517 and 346 $\mu\text{g/kg}$, respectively, with no significant difference between the two ($P > 0.05$). No significant difference in zinc contents in either culture seawater or the whole fish body was observed between the copper-exposed group and the control group ($P > 0.05$) (Table 4).

DISCUSSION

This study shows that infected fish in the copper-exposed group recovered completely, while those in the non-copper-exposed group all died before day 8. According to its life cycle, *C. irritans* produces 300 times more theronts to launch reinfection within 1 week when the surrounding temperature ranges from 27°C to 28°C (1, 6). In this study, both the numbers of *C. irritans* cells and fish deaths suddenly increased in the non-copper-exposed group after 1 week, indicating that a large-scale reinfection sufficient to kill all of the fish had taken place. In contrast, the reductions in the numbers of trophonts and tomonts in the copper-exposed group suggest that the copper-containing surface effectively blocks the infection cycle of *C. irritans* within a

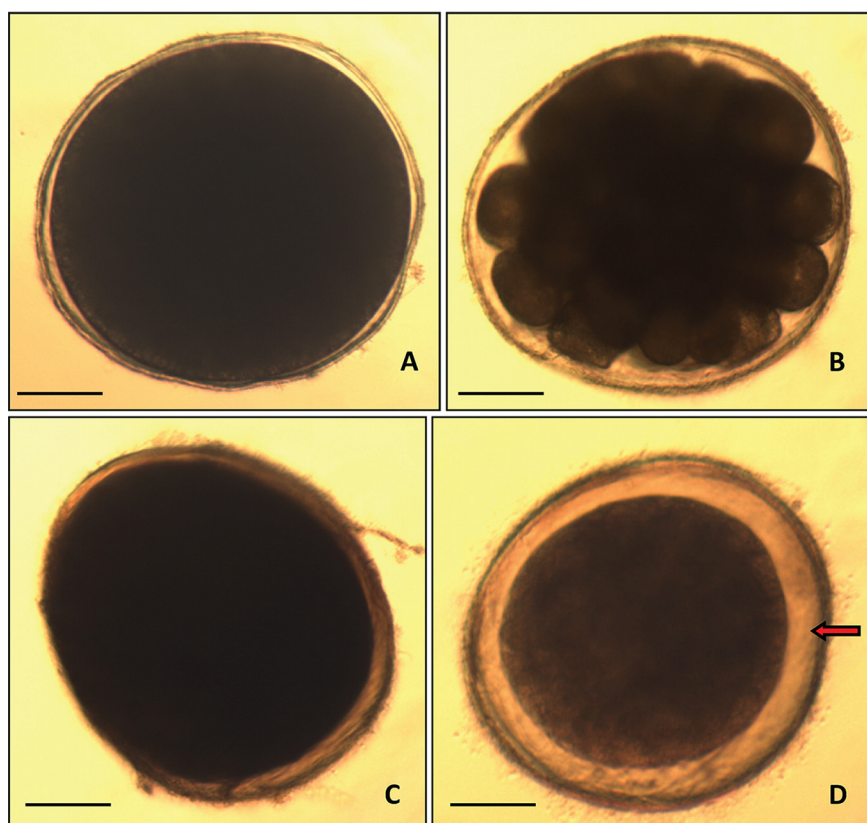


FIG 3 *In vitro* microstructural images of *C. irritans* tomonts. (A) Tomonts were spread on a polystyrene surface and used as the control. (B) Tomonts spread on a polystyrene surface produced 300 times more theront cells after 3 days of incubation in fresh seawater. (C and D) Although cytoplasmic membrane disintegration was not observed in tomonts, tomonts that were in contact with copper alloy sheets for 1 h and were incubated with fresh seawater for 3 days (C) or 14 days (D) more completely lost their capacity for proliferation and eventually died. The arrow indicates the increased gap between the cyst wall and the plasma membrane. Scale bars, 50 μm .

1-week period. Therefore, we may conclude that the copper surface prevents the outbreak of cryptocaryoniasis in the aquaculture of *L. crocea*.

A large number of studies have confirmed that copper surfaces can effectively kill pathogenic bacteria through surface contact (22, 23). Our results prove that sheets of copper alloy containing 74% to 78% copper can also effectively kill *C. irritans*, a fish parasitic ciliate, through surface contact. Copper surfaces kill microorganisms via released copper ions that are taken up by microbial cells through direct contact (23). Copper ions are known to be toxic to bacteria and other microorganisms (18). Thus, the key to successful treatment against *C. irritans* is penetration of the copper ions into parasitic cells. In this study, we assayed the copper ion concentrations in tomonts by inductively coupled plasma mass spectroscopy (ICP-MS) and found that the intracellular copper ion concentration was 100-fold increased after exposure to copper surfaces. As the time of contact between tomonts and copper surfaces increased, copper contents in the tomonts increased significantly, as did tomont mortality rates. The direct correlation between copper ion concentrations and toxicity suggests that copper ions are the major killers of tomonts, which is in agreement with a previous study that showed that increasingly potent bactericidal effects came with increasing copper ion release (23). To our knowledge, this study is the first to report that sheets of copper alloy can kill protozoans, whereas previously characterized eukaryotic death due to contact with copper alloy involved yeast (18) and fungi (24). The tomont is encapsulated in a multilayer cyst wall that is hundreds of times thicker than the cell walls of bacteria (4,000 nm versus 15 to 30 nm) (25). Such a thick cyst wall can effectively block many

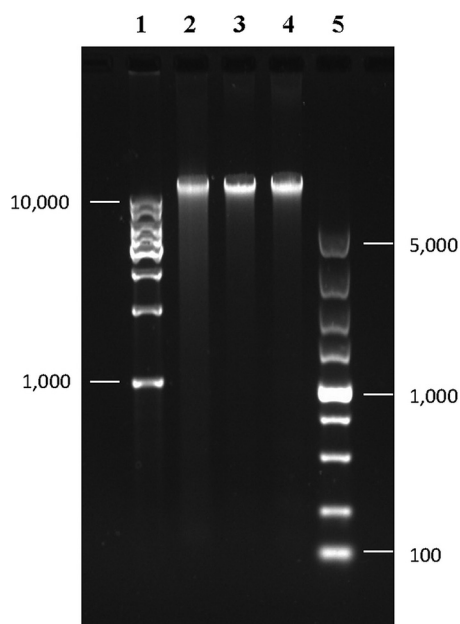


FIG 4 Agarose gel electrophoresis of purified genomic DNA of *C. irritans* tomonts. Lane 2, cells not exposed to copper alloy surfaces; lane 3, cells exposed to a copper alloy sheet for 0.5 h; lane 4, cells exposed to copper for 1 h; lane 1, 1-kb DNA ladder; lane 5, 5-kb DNA ladder.

chemicals, rendering the parasite highly drug resistant. The mechanism through which copper ions penetrate the cyst wall is worthy of further exploration.

Copper ions that enter bacterial cells may lead to cell membrane damage, DNA damage, and cell lysis (26). In this study, the cytoplasmic membrane of the protomonts was ruptured within a few minutes of contact with the copper surface, and there was a large amount of cytoplasmic outflow. Research has shown that copper-induced killing is likely due to membrane damage (27). Tomonts are the most resilient phase in the life cycle of *C. irritans* (28), but our findings showed that copper surface contact was toxic even to tomonts, although no membrane rupture was observed. Based on a previously published study on yeast (18), we assumed that the cyst wall has the function of protecting cell membrane integrity. We also determined that the copper surface did not degrade tomont genomic DNA. Studies have suggested that both lipid peroxidation and cell death occur before genomic DNA degradation, but in some cases genomic DNA is not degraded during the course of cell death (14, 22). This finding indicates that genomic DNA degradation is not the main cause of cell death (14) but may play a role in preventing the horizontal transfer of antibiotic resistance genes in other organisms (13).

Protomonts secrete sticky cyst wall materials to encapsulate themselves to form tomonts, and these materials also allow them to adhere to the bottom of aquaculture tanks (1). In aquaculture, tomonts not only are firmly fixed to the bottom of the container but also are very small, making them difficult to eradicate even with vigorous scrubbing. Furthermore, only a small number of residual tomonts are required for an outbreak. When protomonts fall onto copper surfaces, either they die before secreting cyst walls and exhibit collapsed cytoplasmic membranes and leaky cytoplasm or they gradually die after tomont formation. At that stage, the dead protomonts and tomonts can be easily washed away by seawater. It is important to note that, in areas that are not fully covered by copper alloy sheets, some residual tomonts may survive. *C. irritans* and other fouling organisms cannot adhere to the copper cage or copper coating; therefore, protomonts and tomonts that are shed by infected fish fall through the holes in the cage into the sea. Since *C. irritans* theronts move irregularly, if the distance between the cage and the seabed is sufficiently large, then the risk of reinfection is

TABLE 4 Copper and zinc concentrations in used seawater, whole fish body, and tomonts treated with copper alloy surface

Concn ^a									
Used seawater				Whole fish body			Tomonts		
Ion	Control ($\mu\text{g/liter}$)	Copper alloy ($\mu\text{g/liter}$)	Fold difference	Control ($\mu\text{g/kg}$)	Copper alloy ($\mu\text{g/kg}$)	Fold difference	Control ($\mu\text{g/kg}$)	0.5 h post-copper alloy treatment ($\mu\text{g/kg}$)	1 h post-copper alloy treatment ($\mu\text{g/kg}$)
Cu	0.02 \pm 0.01	3.57 \pm 0.89 ^b	179	346 \pm 119	517 \pm 167	1.49	2.08 \pm 0.02	257 \pm 0.34 ^b	283 \pm 2.80 ^b
Zn	5.06 \pm 2.99	5.11 \pm 1.54	1.00	9,610 \pm 1,519	8,259 \pm 259	0.86	24.10 \pm 0.03	58.59 \pm 0.20 ^b	88.81 \pm 0.02 ^b

^aValues are mean \pm SD ($n = 3$).

^bSignificantly different from the control ($P < 0.05$, Tukey's multiple comparisons).

greatly reduced. This is assumed to be the reason why *C. irritans* outbreaks are not often observed in copper cage aquaculture.

Heavy metal residues in food and pollution of marine environments are unavoidable issues in aquaculture. In addition, flesh can absorb a large amount of copper ions when placed in direct contact with a copper-containing surface (26), with time-dependent concentration increases that can exceed the current United States/Canada-recommended dietary reference intake amount (0.9 mg) (29). In this study, *L. crocea* rarely had direct contact with the copper surface at the bottom, which may be the reason why the fish did not absorb excessive amounts of copper. It is also notable that the copper ions released from the copper sheet into the culture seawater were at low concentrations and bore no threat to the cultured fish or the environment, which is in agreement with a previous publication by Kalantzi et al. (20). Bottom-crawling fish, however, such as *Sebastiscus marmoratus* and *Epinephelus coioides*, should be investigated for excessive copper accumulation in aquaculture when the bottoms of the tanks are covered with copper-containing materials.

In conclusion, a sheet of copper alloy prevents cryptocaryoniasis outbreaks after direct contact with *C. irritans* cells, which is due to copper ion release from the surface of the copper-containing sheets, followed by absorption into *C. irritans* cells. The copper ions are highly toxic and induce permeability in the protomont plasma membrane, thereby inhibiting the secretion of the cyst wall material that allows *C. irritans* cells to adhere to the substrate.

MATERIALS AND METHODS

Parasites and experimental fish. *C. irritans* tomonts were collected from the bottom of tanks containing naturally infected *L. crocea* (150 ± 10 g), and the same fish species was used as the host for the next cycle of *C. irritans* propagation and collection, as described previously (30). In brief, *L. crocea* fish were infected with a nonlethal concentration of theronts ($\leq 10,000$ theronts/fish) in 5 liters of seawater per fish. Two hours after infection, fresh seawater was added. Three days postinfection, white spots were observed on the fins, skin, and gills of the fish. Four days postinfection, large numbers of tomonts were found adhering to the bottom of the tanks. The fish were then transferred to a clean aquarium without parasites. Tomonts were collected by carefully discarding the debris and were incubated in 1-liter beakers. After 2 or 3 days of incubation, up to 300 theronts were released from each tomont. Synchronous theronts were then collected for the propagation of *C. irritans*.

Healthy *L. crocea* fish (6 ± 1 g) were purchased from Fisheries Co., Ltd. (Fuding City, Fujian Province, People's Republic of China). Ten randomly selected fish were examined. No parasites were detected on the gills, fins, or skin of these fish. The fish were overfed twice a day (8:00 and 15:00) with commercial pellet feed.

Seawater was filtered through a sand filter twice before being used in aquaculture. The seawater was oxygenated continuously throughout the experiment and was replaced twice a day (09:00 and 15:00). The seawater salinity, temperature, light intensity, and photoperiod for aquaculture were 29 to 31‰, $28 \pm 1^\circ\text{C}$, 1,000 lx, and 12-h light/12-h dark, respectively.

Copper-containing surfaces. The copper-containing surfaces used in this study were copper alloy sheets (0.5 mm thick) provided by the Copper Co. Ltd. (Luoyang, People's Republic of China) and composed of LC6911 (74% to 78% copper, 17.6% to 24.4% zinc, 1.0% to 3.2% aluminum, 0.3% to 0.9% nickel, and $\leq 0.3\%$ other [wt/wt]). The copper alloy surface was cleaned with a soft cloth and immersed in seawater before use.

Measurement of survival rates, relative infection intensity, and relative protomont and tomont numbers. Active *C. irritans* theronts that had been hatched from tomonts for no more than 2 hours were collected in 1-liter beakers. The concentrations of parasites were calculated as described previously (31). In brief, following agitation to ensure even distribution, 5 drops of a 20- μl suspension of theronts were placed on a clean glass slide. Theronts were immobilized with 20% formalin and counted with a microscope to determine their concentrations.

A total of 540 healthy fish (6 g/fish) were selected randomly for this experiment. A total of 360 fish were infected with theronts at a nonlethal concentration (20 theronts/g of fish) (7) in a 1,062-liter fiberglass aquarium (diameter of the bottom, 130 cm; height, 80 cm), with 1 liter of seawater for each fish. The infection lasted for 2 hours and was performed in the dark. The 360 infected fish were separated randomly into two groups (group B and group C). Each group contained six parallel subgroups (344-liter fiberglass aquarium; diameter of the bottom, 74 cm; height, 80 cm), and each aquarium contained 30 fish. Since the protomonts and tomonts attach to the bottoms of the aquaculture tanks, the bottoms of the tanks in group C were covered with copper alloy sheets; the bottoms of the tanks in group B were fiberglass with no copper alloy sheets. Another 180 uninfected fish were used as the control group (group A) and were treated in the same way as the infected fish. Similarly, the control group contained six parallel subgroups (30 fish/aquarium), with no copper alloy sheets covering the bottoms of the tanks. Three of the aforementioned subgroups were used to measure survival rates and the numbers of tomonts, while the other three were used to measure RII.

Every day, we recorded the number of dead fish in each aquarium and calculated the survival rate as follows: survival rate (%) = $100 \times \text{number of surviving fish} / \text{initial number of fish}$. Every 24 h, the trophonts on the left pectoral fin were counted under an inverted microscope (Olympus IX70; Olympus Optical, Tokyo, Japan). RII was calculated as the number of trophonts on the left pectoral fin/weight of the fish (g). Every 24 h, we recorded the number of tomonts from each tank in 3 polystyrene petri dishes (diameter, 8.5 cm). For group C, the dishes were covered with copper alloy sheets; for group B, no copper alloy sheets were applied. The RTN and relative protomont number (RPTN) were calculated as follows: $\text{RTN (or [RPTN])} = [S_{\text{tank}} / (3 \times S_{\text{dish}})] \times \text{total number of tomonts (or protomonts) in 3 petri dishes/fish number/average weight per fish (g)}$ ($S_{\text{dish}} = 56.75 \text{ cm}^2$ and $S_{\text{tank}} = 4,300.84 \text{ cm}^2$) (where S_{tank} is the area at the bottom of the 344-liter fiberglass aquarium and S_{dish} is the area at the bottom of the polystyrene petri dish).

Samples were collected from groups B and C as well as the control group (group A) at 15 d postinfection. Three fish and 1 liter of seawater were randomly removed from each aquarium and immediately stored at -20°C until analyses of copper and zinc ion concentrations were performed.

In vitro contact-based killing assays on copper surfaces. Newly formed protomonts or tomonts were collected and spread onto the surfaces of the copper alloy sheets. For each treatment, 3 replicates were performed, and 100 cells assigned to each replicate were observed by microscopy. Since the cytoplasm of tomonts is encapsulated by a cyst wall, the cell membrane does not rupture as readily as that of protomonts, making it difficult to determine immediately whether the tomont cells have died. Based on the life cycle of *C. irritans*, if the newly formed tomont has not divided after $3 \text{ days at } 28 \pm 1^\circ\text{C}$, then it can be concluded that it has died (Fig. 1). Some of the treated tomonts were transferred into fresh seawater to assess their survival by observing cell division or hatching of theronts at 72 h and 14 d under an inverted microscope. Other newly formed protomonts and tomonts were spread on a polystyrene surface and used as controls. These protomonts and tomonts were treated in the same way as the copper alloy treatment group.

At 0.5 h and 1 h, tomonts were removed from copper surfaces and residual copper was removed by washing with sterilized seawater. Some of the tomont cells were immediately sampled and stored at -20°C until they were used for genomic DNA gel electrophoresis and copper concentration assays.

ICP-MS analysis. Copper concentrations in aquaculture seawater, tomonts, and the entire *L. crocea* body were analyzed using a protocol modified from that described by Espírito Santo et al. (27). Briefly, samples were acid mineralized using nitric acid at a final concentration of 5% (vol/vol). As an internal standard, gallium [$\text{Ga}(\text{NO}_3)_3$] was added to a final concentration of 50 ppb. Elemental analysis was performed using an Agilent model 7700 ICP-MS system (Agilent, Santa Clara, CA USA) operating with a collision cell with flow rates of 3.5 ml/min H_2 and 1.5 ml/min He.

Gel electrophoresis of *C. irritans* tomont genomic DNA. DNA from the aforementioned samples was purified using the SH09-D kit (Sanhoo), and genomic DNA was separated, along with 10-kb and 5-kb markers, on a 0.8% agarose gel (HydraGene) containing GelRed DNA stain (Biotium). The gel was placed under a voltage of 115 V for 40 min. Gels were observed with a UV light box and photographed using GeneScan software.

Data analysis and statistical methods. Statistical analyses were performed using SPSS 11.5. One-way analysis of variance (ANOVA) was used to compare the survival rates, RTN, RII, and patterns of copper and zinc concentrations in tomonts, used seawater, and fish flesh after culture in tanks containing copper alloy sheets. If a significant difference was detected, then *post hoc* multiple-comparison procedures (Tukey's test) were performed. Data are presented as the mean \pm standard deviation (SD). *P* values of <0.05 were considered statistically significant.

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